CLONING OF A GENE MUTATION FOR PARKINSON'S DISEASE

BACKGROUND OF THE INVENTION

1. Field of the Invention

Parkinson's disease (PD) is a common neurodegenerative disorder with a lifetime incidence of approximately 2 percent. A pattern of familial aggregation has been documented for the disorder, and it was recently reported that a PD susceptibility gene in a large Italian kindred is located on the long arm of human chromosome 4. We have identified a mutation in the alpha synuclein gene, which codes for a presynaptic protein thought to be involved in neuronal plasticity, in the Italian kindred and in three unrelated families of Greek origin with autosomal dominant inheritance for the PD phenotype. This finding of a specific molecular alteration which is causative for PD will permit the detailed understanding of the pathophysiology of the disorder. In addition, methods of screening nucleic acids for the presence of mutations in the synuclein gene to test for predisposition to Parkinson's Disease are now possible.

2. Technology Background

Parkinson's disease (PD) was first described by James Parkinson in 1817 (*1*). The clinical manifestations of this neurodegenerative disorder include resting tremor, muscular rigidity, bradykinesia and postural instability. A relatively specific pathological feature accompanying the

neuronal degeneration is the intracytoplasmic inclusion body, known as the Lewy body, which is found in many regions including the substantia nigra, locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei, and the central and peripheral divisions of the autonomic nervous system (*1*).

In many cases a heritable factor predisposes to the development of the clinical syndrome (*2*). We have recently shown that genetic markers on human chromosome 4q21-q23 segregate with the PD phenotype in a large family of Italian descent (*3*). The clinical picture of the PD phenotype in the Italian kindred has been well documented to be typical for PD, including Lewy bodies, with the exception of a relatively earlier age of onset of illness at 46 ± 13 years. In this family the penetrance of the gene has been estimated to be 85%, suggesting that a single gene defect is sufficient to determine the PD phenotype.

We now report the identification of a mutation in the alpha synuclein gene that is associated with Parkinson's disease. The mutation, an Ala53Thr substitution, was found to be linked to the PD phenotype in four independent PD families and absent from 314 control chromosomes, providing strong genetic evidence that this mutation in the human alpha synuclein gene is causative for the PD phenotype in these families.

The Ala53Thr substitution is localized in a region of the protein whose secondary structure predicts an alpha helical formation, bounded by beta sheets. Substitution of the alanine with threonine is predicted to disrupt the alpha

helix and extend the beta sheet structure. Beta pleated sheets are thought to be involved in the self aggregation of proteins which could lead to the formation of amyloid like structures (6).

This was already tested in the case of NAC35, the 35 amino acid peptide derived from alpha-synuclein that was first isolated from plaques found in patients with Alzheimer's disease (4). NAC35 was shown to self aggregate and form amyloid fibril which shared the `amyloid' characteristics of insolubility in aqueous solutions and green birefringence under polarized light, subsequent to Congo red staining (6). NAC35 is located in the middle of the alpha synuclein molecule and extends from amino acid 61 to amino acid 95. Residue 53, which is found to be mutated in PD, is outside the NAC35 peptide found in amyloid plaques. However, the true size of the NAC peptide involved in the plaques is not known since the protease used to isolate the peptide from AD tissue cuts at lysine 60 of the alpha synuclein protein. It is therefore possible that amino acid 53 may be part of the NAC peptide found in plaques. In crosslinking experiments with beta amyloid (Abeta), it was demonstrated (6) that residues 1-56 and 57-97 specifically bind amyloid and that a synthetic peptide consisting of residues 32-57 performed similarly.

Three members of the synuclein family have been characterized in the rat, with SYN1 exhibiting 95% homology with the human alpha-synuclein protein (7). SYN 1 of the rat is expressed in many regions of the brain, with high levels found in the olfactory bulb and tract, the hippocampus,

dentate gyrus, habenula, amygdala and piriform cortex, and with intermediate levels in the granular layer of the cerebellum, substantia nigra, caudate-putamen, and dorsal raphe (7). This pattern of expression coincides with the distribution of the Lewy bodies found in brains of patients with Parkinson's disease. It is also interesting to note that decrease in olfactory sense often accompanies the syndromic features of Parkinson's disease, and in many cases it is proposed that hyposmia is a prodromic sign of the illness (8).

In the zebra-finch the homologue to alpha synuclein, synelfin, is thought to be involved in the process of song learning, suggesting a role for synuclein perhaps in memory and learning (9). In contrast to humans, rats have a threonine at residue 53 of their homologues to the human alpha synuclein gene (Figure 4). Similarly, the zebra-finch synelfin carries a threonine at amino acid 53, whereas both Bos taurus and Torpedo californica do not (10). There are no reports that suggest the presence of Lewy bodies in the brains of the rat or the zebra finch or a phenotype resembling that of PD. Lack of any phenotype could be explained by a combination of factors, including the following: the relative short life span of rodents may prohibit the observation of a late onset disorder, interaction with other cellular components not present in the rat may be required for the phenotype, absence of a critical environmental trigger in the rodents, or finally a heterozygous status Ala/Thr may be necessary for the production of a phenotype.

Studies of early onset AD have previously documented that missense mutations can cause an adult onset neurodegenerative disorder. Of the 31 mutations described so far in the loci for presenilin 1 and 2, thirty were missense and one was a splice variant (11). Missense mutations in the prion protein have also been implicated in the amyloid production seen in Gerstmann-Straüssler-Scheinker and Creutzfeld-Jakob diseases, both forms of spongiform encephalopathy (12). Studies in these neurodegenerative disorders have pointed to the importance of the physical chemical properties of mutant cellular proteins in initiating and propagating neuronal lesions leading to disease. Similar studies in the synuclein protein family may provide valuable insights into the etiology and pathogenesis of PD.

Similarly with the mutations in the presenilin genes in patients with early onset Alzheimer's disease, the mutation identified in the alpha synuclein gene is unlikely to account for the majority of sporadic and familial cases of PD. However, this mutation may account for a significant proportion of those families with a highly penetrant, early onset autosomal dominant PD phenotype.

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

3. Summary of the Invention

As described herein, we have discovered that particular mutations in the alpha synuclein gene are associated with predisposition to Parkinson's disease. Accordingly, the present invention includes an isolated nucleic acid comprising a mutated synuclein gene. In particular, the isolated nucleic acid of the present invention contains at least one mutation in the alpha synuclein gene at base pair position 209 of Genbank # L08850, which, in particular, is a change from guanine to adenine. However, since other mutations in the alpha synuclein gene may also lead to Parkinson's Disease (PD), other mutations are also included. In addition, it is conceivable that mutations in the related beta (46)(SEQ ID NO 11) and gamma (SEQ ID NOs 12 and 13) synuclein genes may also lead to PD. Thus, mutated homologues of the alpha synuclein gene are also included in the present invention. Vectors comprising the isolated nucleic acid and host cells comprising such vectors are included as well.

Knowledge of particular genes that are associated with PD allows for the search for other specific PD mutations. Accordingly, the present invention also includes a method of using a synuclein gene sequence to identify specific PD mutations. Such mutations may occur in an unrelated population or in a family that demonstrates passage of PD within the family tree.

Since knowledge of mutations associated with Parkinson's disease allows the development of genetic screens that test for an individual's chances of being predisposed to the disease, and such tests may be performed by hybridization

analysis using oligonucleotides complementary to the sequence of interest or by PCR amplification using oligonucleotides that are complementary to sequences flanking the mutation, the present invention also includes oligonucleotides complementary to a portion of the synuclein gene, wherein said portion comprises or flanks a mutation associated with predisposition to Parkinson's Disease. In particular, the oligonucleotides of the present invention will have a sequence that is complementary to a sequence from the alpha synuclein gene that includes or flanks base pair position 209. And in particular, this mutation is a change from guanine to adenine at this position.

Vectors comprising an isolated nucleic acid encoding a mutated synuclein gene will allow the production and isolation of the mutant protein in an appropriate host cell using techniques well known in the art. Alternatively, peptides may be chemically synthesized using techniques also well known in the art. Isolation of such a protein or peptides thereof will allow the study of the molecular mechanisms which lead to development of Parkinson's disease. Accordingly, the present invention also includes an isolated synuclein protein or peptide containing at least one mutation. In particular, this mutation is at a position corresponding to the fifty-third amino acid in the native alpha synuclein protein, and in particular, this mutation is an alanine to threonine substitution.

Peptides corresponding to portions or the entirety of a synuclein gene may be useful as drugs for inhibiting the self-aggregation of mutant proteins that is thought to lead

to Parkinson's disease. Accordingly, the present invention includes a method of testing peptides and other compounds for the ability to interfere with this self-aggregation. Self-aggregation can be tested using a number of established methods, including Congo red staining, electron microscopy pictures of amyloid fibrils, and circular dichroism (CD) spectrophotometry. Using a peptide derived from the alpha synuclein protein that includes the mutant THR amino acid at position 53 alone or in combination with a normal peptide may allow testing for drugs that can inhibit the aggregation or dissolve an aggregate. This procedure can be used to rapidly identify agents that could be used in animal studies, clinical trials, or as diagnostic tools.

Possession of isolated synuclein proteins or peptides will also allow the isolation of specific antibodies using techniques well known in the art. Such antibodies may distinguish a mutant synuclein protein from its wildtype counterpart, and therefor could also be used in diagnostic screens. Alternatively, such antibodies may also be used to inhibit the self-aggregation of proteins during the progression of Parkinson's disease. Accordingly, the present invention also includes antibodies specific for a mutated synuclein protein or peptide. It should be understood that useful derivatives of such antibodies, such as Fv fragments and Fab fragments, are also included.

The above aspects of the present invention will allow methods of detecting subjects at increased risk for Parkinson's Disease. Such a method comprises obtaining a sample comprising nucleic acids from the subjects, and

detecting in the nucleic acids the presence of a mutation which is associated with Parkinson's disease. In particular, the mutation detected by the method of the present invention is located on human chromosome four, preferably in the alpha synuclein gene. In particular, the mutation causes an amino acid substitution at position 53 of the alpha synuclein gene, which is, in particular, an alanine to threonine substitution.

The detecting step of the method of the present invention may be accomplished several different ways as will be described in further depth below. All such methods are well known to those of ordinary skill in the art.

For instance, said detecting step may comprise combining a nucleotide probe which selectively hybridizes to a nucleic acid containing a mutation associated with a predisposition to Parkinson's disease, and detecting the presence of hybridization. Such a probe may be an oligonucleotide that is complementary to a portion of the synuclein gene, wherein said portion comprises the mutation. In particular, such an oligonucleotide is complementary to a mutated alpha synuclein gene having at least one mutation at base pair position 209. In particular, this mutation is a change from guanine to adenine.

The detecting step of the method of the present invention may also comprise amplifying a nucleic acid product comprising said mutation, and detecting the presence of said mutation in the amplified product using any nucleic acid sequencing procedure known in the art. Alternatively, the detecting step may comprise selectively amplifying a nucleic

acid product comprising said mutation, and detecting the presence of amplification using any appropriate method known in the art. Such methods include gel electrophoresis of amplified nucleic acids, and detection of radiolabeled amplified nucleic acids using autoradiographic film or any other detection method known in the art.

The amplifying step of the present invention may be performed using the polymerase chain reaction (PCR), reverse transcriptase PCR (RTPCR), or any other type of PCR reaction known in the art. Accordingly, such a step will comprise at least one annealing step whereby at least one oligonucleotide is annealed to said sample of nucleic acids. In particular, said amplifying step uses two oligonucleotides. And in particular, the two oligonucleotides have the sequences given in SEQ ID NOs 2 and 3.

Alternatively, the detecting step of the method of the present invention comprises detecting the presence or absence of a restriction endonuclease site as detected by enzymatic digest of a nucleic acid sample. Such a detecting means will be possible when a mutation associated with a predisposition to Parkinson's disease results in a sequence having a new restriction endonuclease cleavage site, or loss of a native restriction endonuclease site. In particular, the mutation associated with Parkinson's disease results in the formation of a non-native *Tsp*45I restriction endonuclease site.

Alternatively, the detecting step of the present invention may be performed using a gene-specific primer and subsequent chain termination at the position of the mutation using DNA polymerase and labeled nucleotides or dideoxynucleotides.

The presence of nucleic acids in which a dideoxynucleotide corresponding to the mutation of interest is incorporated at the appropriate position may be detected by any means known in the art, including detection of radiolabeled dideoxynucleotides using, for example, autoradiographic film, or detection of fluorescently-labeled dideoxynucleotides.

Since the methods and compounds of the present invention will be useful in diagnostic screening procedures aimed at identifying individuals having a predisposition for Parkinson's disease, the present invention also includes diagnostic kits which include the compounds of the present invention in a form that allows such compounds to be used quickly and easily for the designated purpose.

Finally, the inventors also contemplate that the isolated nucleic acid, oligonucleotides and antibodies of the present invention may eventually be used in methods directed at the correction or suppression of Parkinson's disease. For example, oligonucleotides or expression vectors designed from the synuclein nucleic acid sequences of the present invention may one day be used in antisense therapy directed at inhibiting expression of the mutated synuclein protein in patients with Parkinson's disease, or in individuals having a predisposition for Parkinson's disease. Similarly, antibodies specific for the mutated synuclein protein may be useful in therapies directed at inhibiting the self-aggregation of mutated proteins or peptides in patients having Parkinson's disease. Knowledge of gene(s) associated with the development of Parkinson's disease may also allow the design of transgenic animals which express the mutant

gene(s). Such animals may serve as a useful disease model, allowing one to test the effects of candidate therapies and therapeutic compositions in the treatment or inhibition of Parkinson's disease.

A detailed description of the present invention is now provided, and should not be considered as limiting on the present invention as described above.

4. Brief Description of the Drawings

Figure 1.

DNA sequence of the PCR product used for mutation detection (SEQ ID NO 1). Oligonucleotide primers are shown by arrows and the numerals 3 and 13 (SEQ ID NO 2 and 3). Intron sequence is shown in lower case and exon sequence in upper case. Amino acid translation of the exon is shown below the DNA sequence. The circled base represents the G209A change in the mutant allele. The resulting amino acid Ala53Thr change is represented by the circled amino acid. The newly created *Tsp*45 I site is indicated above the DNA sequence.

Figure 2.

Mutation analysis of the G209A change is shown in a subpedigree of the Italian kindred. Filled symbols represent affected individuals. Numerical identifiers, denote the individuals immediately above. *Tsp*45 I digestion of PCR products is shown at the bottom of the figure, and fragment sizes are indicated on the right in base pairs.

Figure 3.

Mutation analysis of the G209A change in RT PCR products (7). Lane 1: 100 bp ladder, lanes 2 and 3 normal control, lanes 4 and 5 PD patient, lane 6 negative control without RT enzyme. Sizes are indicated on the right in base pairs. Lanes 2 and 4 show uncut DNA and lanes 3 and 5 show DNA cut with *Tsp*45 I.

Figure 4.

Sequence alignments of alpha synuclein homologues in different species. Accession numbers for the sequences used were as follows: Homo sapiens Swiss-Prot P37840 (SEQ ID NO 4), Rattus norvegicus Swiss-Prot P37377 (SEQ ID NO 5), Bos taurus Swiss-Prot P33567 (SEQ ID NO 6), Serinus canaria genbank L33860 (SEQ ID NO 7), Torpedo californica Swiss-Prot P37379 (SEQ ID NO 8). Numbering on top of the alignments is according to the human sequence. Amino acid 53, which is the site of the Ala53Thr change, is circled.

Figure 5.

The pedigree of a large family with PD (3). The clinical and pathological features of some members of this kindred were previously reported.

Figure 6.

Multipoint LOD score analysis between chromosome 4q markers and the PD locus.

Figure 7.

A table of human synuclein clones identified from various databases. Columns labeled 5' and 3' show the sequence acquisition numbers. Clones were identified by homology to protein or nucleic acid sequence. Human gamma clones were identified by homology to known mouse and rat gamma synuclein sequences.

Figure 8.

Sequence of BAC clone 139A20 for human beta synuclein. BAC clone was isolated using primers to known database sequences described in Figure 7. The sequence shown includes all coding exon sequences and some non-coding intronic sequences.(SEQ ID NO:11)

Figure 9.

Sequence from the 5' end of BAC clone 174P13 for human gamma synuclein. The BAC clone was isolated with primers from the database sequences described in Figure 7.(SEQ ID NO:12)

Figure 10.

Sequence from the 3' end of BAC clone 174P13 for human gamma synuclein. BAC clone was isolated as described in Figure 9. The entire human gamma synuclein gene has now been sequenced and has been deposited in GenBank: accession number AF044311.(SEQ ID NO: 13)

Figure 11.

Sequence of exons 1-7 of the human alpha synuclein gene, plus some flanking intronic sequence for each exon. (SEQ ID NOs 14-19)

5. Detailed Description of the Invention

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

This invention provides a method of diagnosing or predicting a predisposition to Parkinson's disease. The method comprises detecting in a sample from a subject the presence of a mutation, for example, in nucleotide position 209 of the human alpha synuclein gene. The presence of the mutation indicates the presence of or a predisposition to Parkinson's disease.

As used herein, the term "gene" primarily relates to a coding sequence, but can also include some or all of the surrounding or flanking regulatory regions or introns. The term "gene" specifically includes artificial or recombinant genes created from cDNA or genomic DNA, including recombinant genes based upon splice variants.

As used herein, the term "synuclein" gene or protein may refer to the alpha synuclein gene or any homologue thereof.

A "homologue" is understood to mean any related gene or protein that is at least 25% homologous to the alpha synuclein gene or protein or performs a related function. Preferably, a synuclein gene or protein refers to alpha, beta or gamma synuclein, but most preferably refers to alpha synuclein.

As used herein, an "isolated nucleic acid" is a ribonucleic acid, deoxyribonucleic acid, or nucleic acid analog comprising a polynucleotide sequence that has been isolated or separated from sequences that are immediately contiguous, i.e. on the 5' and 3' ends, in the naturally occurring genome of the organism from which it is derived. The term therefor includes, for example, a recombinant nucleic acid which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule independent from any other sequences.

An isolated nucleic acid of the present invention may be "operatively linked" to an expression control sequence or regulatory region. As used herein, "operatively linked" means that the components are joined in such a way that the expression, transcription or translation of the sequence is under the influence or control of the regulatory region.

As used herein, a "predisposition" to Parkinson's disease means an increased probability of developing Parkinson's disease during the subject's lifetime as compared to the average individual.

Pertaining to this probability, a LOD score is a measure of genetic linkage used herein, defined as the $\log_{10}$ ratio of

the probability that the data would have arisen if the loci are linked to the probability that the data could have arisen from unlinked loci. The conventional threshold for declaring linkage is a LOD score of 3.0, that is, a 1000:1 ratio (which must be compared with the 50:1 probability that any random pair of loci will be unlinked).

As used herein, reference to "base pair position" or "amino acid position" when referring to an isolated nucleic acid, probe, protein or peptide always indicates the relative position in the native gene or protein.

A "probe" refers to a nucleic acid which has sufficient nucleotides surrounding the codons at the mutation positions to distinguish the nucleic acid from nucleic acids encoding non-related genes. The specific length of the nucleic acid is a matter of routine choice based on the desired function of the sequence. For example, if one is making probes to detect the mutation in base pair position 209, the length of the nucleic acid is preferably small, but must be long enough to prevent hybridization to undesired background sequences. However, if the desired hybridization is to a nucleic acid which has been amplified, background hybridization is less of a concern and a smaller probe can be used. In general, such a probe will be between 10 and 100 nucleotides, especially between 10 and 40 and preferably between 15 and 25 nucleotides in length. It is apparent to one of skill in the art that nucleotide substitutions, deletions, and additions may be incorporated into the polynucleotides of the invention. However, such nucleotide substitutions, deletions, and additions should not substantially disrupt the ability of

the polynucleotide to hybridize under conditions that are sufficiently stringent to result in specific hybridization.

As used herein with respect to genes, "the term "normal" refers to a gene which encodes a normal protein. As used herein with respect to proteins, the term "normal" means a protein which performs its usual or normal physiological role and which is not associated with, or causative of, a pathogenic condition or state. Therefor, the term "normal" is generally synonomous with the phrase "wild type".

For any given gene or corresponding protein, a multiplicity of normal allelic variants may exist, none of which is associated with the development of a pathogenic condition or disease state. Such normal allelic variants include, but are not limited to, variants in which one or more nucleotide substitutions do not result in a change in the encoded amino acid sequence.

As used herein, the term "mutation" generally refers to a mutation in a gene that is associated with a predisposition to Parkinson's disease. "Mutant" can specifically refer to a mutation at nucleotide position 209 of the synuclein gene, and is in particularly a G to A transition. However, other mutations in the synuclein gene or other genes which are associated with a predisposition to Parkinson's disease are also encompassed. Furthermore, the term "mutation" is not limited to transition mutations, but can also mean a deletion, insertion or transversion as well.

The term "mutant", as it applies to synuclein genes, is not intended to embrace sequence variants which, due to the degeneracy of the genetic code, encode proteins identical to

the normal sequences disclosed or otherwise enabled herein; nor is it intended to embrace sequence variants which, although they encode different proteins, encode proteins which are functionally equivalent to normal synuclein proteins. The term "mutant" means a protein which does not perform its usual or normal physiological role and which is associated with, or causative of, a pathogenic condition or state.

Since a mutation can be a substitution, deletion or insertion, a mutated synuclein "protein" is understood to refer to the amino acid sequence resulting from any such mutation whether the resulting protein is shorter, longer or modified, i.e. due to an alteration in reading frame or generation of stop codon. In addition, "peptide" is understood to refer to a portion of the mutated protein that is preferably at least five base pairs long, and more preferably at least 10 base pairs long. This portion may be derived from the amino or carboxyl terminus, or it may be an internal portion of the full length protein. As such, a peptide may be chemically synthesized using any method known in the art, or may be made using a recombinant DNA technology and an appropriate purification scheme or isolated from the native protein using enzymatic digestion.

As used herein, the term "substantially pure" means a preparation which is at least 60% by weight the compound of interest. Preferably the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any

appropriate method, i.e. column chromotography, gel electrophoresis or HPLC analysis.

"Specific or selective hybridization" as used herein means the formation of hybrids between a probe nucleic acid (e.g., a nucleic acid which may include substitutions, deletions, and/or additions) and a specific target nucleic acid (e.g., a nucleic acid having the mutated sequence), wherein the probe preferentially hybridizes to the specific target such that, for example, a band corresponding to the mutated DNA or restriction fragment thereof can be identified on a Southern blot, whereas a corresponding normal or wild-type DNA is not identified or can be discriminated from a variant DNA on the basis of signal intensity. Hybridization probes capable of specific hybridization to detect a single-base mismatch may be designed according to methods known in the art (13-17).

"Stringent" as it refers to hybridization conditions is a term of art understood by those of ordinary skill to refer to those conditions of temperature, chaotrophic acids, buffer and ionic strength which permit hybridization of a particular nucleic acid sequence to its complementary sequence and not to substantially different sequences. The exact conditions which constitute "stringent" conditions depend on the nature of the nucleic acid sequence, the length of the sequence, and the frequency of occurrence of subsets of that sequence within other non-identical sequences. By varying hybridization conditions from a level of stringency at which non-specific hybridization occurs to a level at which only specific hybridization occurs, one of ordinary skill in the art can, without undue experimentation, determine conditions

which will allow a given sequence to hybridize only with complementary sequences.

Suitable ranges of stringency conditions are described in Sambrook et al. (13). Hybridization conditions, depending on the length and commonality of a sequence, may include temperatures of 20_C-65_C and ionic strengths from 5X to 0.1X SSC. Highly stringent hybridization conditions may include temperatures as low as 40_C-42_C (when denaturants such as formamide are included) or up to 60_C-65_C in ionic strengths as low as 0.1X SSC. These ranges are, however, only illustrative and, depending on the nature of the target sequence, and possible future technological developments, may be more stringent than necessary. Appropriate conditions may be determined for each specific nucleic acid sequence or oligonucleotide probe using standard control and a level of experimentation that is not considered to be undue by those of skill in the art.

As discussed below in greater detail, the mutation can be detected by many methods. For example, the detecting step can comprise combining a nucleotide probe capable of selectively hybridizing to a nucleic acid containing the mutation with a nucleic acid in the sample and detecting the presence of hybridization. Additionally, the detecting step can comprise amplifying the nucleotides surrounding and including the mutation and detecting the presence of the mutation in the amplified product, or selectively amplifying the nucleotides

of the mutation and detecting the presence of amplification. Finally, the detecting step can comprise detecting the presence or absence of a restriction fragment created by an enzyme digest of the sample nucleic acid, or any other detection means known in the art.

Detection Techniques

Once the location of a PD-relevant mutation is known, the methods to detect such a mutation are standard in the art. The sequence of various nucleotide probes can be determined from the known sequence of the relevant gene, especially the sequences surrounding the mutation.

Detection of point mutations using direct probing involves the use of oligonucleotide probes which may be prepared, for example, synthetically or by nick translation. The probes may be suitably labeled using, for example, a radio label, enzyme label, fluorescent label, biotin-avidin label and the like for subsequent visualization by any appropriate assay, i.e. Southern blot hybridization. In this procedure, the labeled probe is reacted with sample DNA that is bound, for example, to a nylon filter under conditions such that only fully complementary sequences hybridize. The areas that carry DNA sequences complementary to the labeled DNA probe become labeled themselves as a consequence of the reannealing reaction. The areas of the filter that exhibit such labeling may then be visualized, for example, by autoradiography.

Methods of manipulating hybridization conditions to achieve varying degrees of specificity are well known in the art. For example, tetra-alkyl ammonium salts may be used to

bind selectively to A-T base pairs, thus displacing the dissociation equilibrium and raising the melting temperature. At 3M Me 4NCl, this is sufficient to shift the melting temperature to that of G-C pairs. This results in a marked sharpening of the melting profile. The stringency of hybridization in such an experiment is usually 5_C below the Ti (the irreversible melting temperature of the hybrid formed between the probe and its target sequence) for the given chain length. For a 20mer oligonucleotide probe, the recommended hybridization temperature is about 58_C. The washing temperatures are unique to the sequence under investigation and need to be optimized for each variant.

There are certainly other ways known in the art for adjusting hybridization conditions in view of desired specificity. For instance, although hybridization may be carried out in accordance with conventional hybridization methods under suitable conditions with respect to e.g. stringency, incubation time, temperature, etc, the choice of conditions will depend on the desired degree of complementarity between the fragments to be hybridized. A high degree of complementarity requires more stringent conditions such as low salt concentrations, low ionic strength of the buffer and higher temperatures, whereas a low degree of complementarity requires less stringent conditions, e.g. higher salt concentration, higher ionic strength of the buffer or lower temperatures, for the hybridization to take place.

The support to which DNA or RNA fragments of the sample to be analyzed are bound in denatured form is preferably a solid

support and may have any convenient shape. Thus, it may, for instance, be in the form of a plate, e.g. a thin layer or a microtiter plate, a strip, a solid particle e.g. in the form of a bead such as a latex bead, a filter, a film or paper. The solid support may be composed of a polymer, preferably nylon or nitrocellulose.

Alternative probing techniques, such as ligase chain reaction (LCR), may involve the use of mismatch probes, i.e., probes which are fully complementary with the target except at the point of the mutation. The target sequence is then allowed to hybridize both with oligonucleotides which are fully complementary and have oligonucleotides containing a mismatch, under conditions which will distinguish between the two. By manipulating the reaction conditions according to the above considerations, it is possible to obtain hybridization only where there is full complementarity. If a mismatch is present there is significantly reduced hybridization.

The polymerase chain reaction (PCR) is a technique that amplifies specific DNA sequences with remarkable efficiency. Repeated cycles of denaturation, primer annealing and extension carried out with Taq polymerase, e.g., a heat stable DNA polymerase, leads to exponential increases in the concentration of desired DNA sequences. Given a knowledge of the nucleotide sequence of the mutations, synthetic oligonucleotides can be prepared which are complementary to sequences which flank the DNA of interest. Each oligonucleotide is complementary to one of the two strands. The DNA is denatured at high temperatures (e.g., 95_C) and then reannealed in the presence of a large molar excess of

oligonucleotides. The oligonucleotides, oriented with their 3' ends pointing towards each other, hybridize to opposite strands of the target sequence and prime enzymatic extension along the nucleic acid template in the presence of the four deoxyribonucleotide triphosphates. The end product is then denatured again for another cycle. After this three-step cycle has been repeated several times, amplification of a DNA segment by more than one million-fold can be achieved. The resulting DNA may then be directly sequenced in order to locate any genetic alteration. Alternatively, it may be possible to prepare oligonucleotides that will only bind to altered DNA, so that PCR will only result in multiplication of the DNA if the mutation is present. Following PCR, direct visualization or allele-specific oligonucleotide hybridization (18) may be used to detect the Parkinson's disease point mutation. Alternatively, PCR may be followed by restriction endonuclease digestion with subsequent analysis of the resultant products.

As shown in the examples, the substitution of G for A at base pair 209 of the synuclein, results in the gain of a *Tsp*45I site. The gain of this restriction endonuclease recognition site facilitates the detection of the Parkinson's disease mutation using restriction fragment length polymorphism (RFLP) analysis or by detection of the presence or absence of the restriction site in a PCR product that spans base pair position 209.

For RFLP analysis, DNA is obtained, for example from the blood cells of the subject suspected of having Parkinson's disease and from a normal subject, is digested with a

restriction endonuclease, and subsequently separated on the basis of size by agarose gel electrophoresis. The Southern technique can then be used to detect, by hybridization with labeled probes, the products of endonuclease digestion. The patterns obtained from the Southern blot can then be compared. Using such an approach, an additional restriction endonuclease site, such as a *Tsp*45I site, is detected by determining the number of bands detected and comparing this number to the normal subject.

The creation of a new restriction site as a result of a nucleotide substitution at a disclosed mutation site can be readily determined by reference to the genetic code and a list of nucleotide sequences recognized by restriction endonucleases (19).

In general, primers for PCR are usually about 20 bp in length, and are most preferably 15-25 bp. Denaturation of strands usually takes place at 94_C. and extension from the primers is usually at 72_C. The annealing temperature varies according to the sequence under investigation. Examples of reaction times are: 20 mins denaturing; 35 cycles of 2 min, 1 min, 1 min for annealing, extension and denaturation; and finally a 5 min extension step.

PCR "amplification of specific alleles" (PASA) may also be used to detect the presence of the PD mutation. PASA is a rapid method of detecting single-base mutations or polymorphisms (22-28). PASA (also known as allele specific amplification) involves amplification with two oligonucleotide primers such that one is allele-specific. The desired allele is efficiently amplified, while the other

allele(s) is poorly amplified because it mismatches with a base at or near the 3' end of the allele-specific primer. Thus, PASA or the related method of PAMSA may be used to specifically amplify the mutation sequences of the invention. Where such amplification is done on genetic material (or RNA) obtained from an individual, it can serve as a method of detecting the presence of the mutations.

As mentioned above, a method known as ligase chain reaction (LCR) can be used to successfully detect a single-base substitution (29, 30). LCR probes may be combined or multiplexed for simultaneously screening for multiple different mutations. Thus, LCR can be particularly useful where multiple mutations are predictive of the same disease.

Finally, the Parkinson's disease mutation of the present invention may also be detected using chain termination with labeled dideoxynucleotides. For instance, U.S. Patent No. 5,047,519 to Hobbs et al. discloses fluorescently-labeled nucleotides as chain-terminating substrates for a fluorescence-based DNA sequencing method. With such substrates and knowledge of the gene sequence of interest, it is possible to design an assay using a gene-specific primer to initiate a polymerase reaction immediately flanking the position of the mutation, employing color-coded dideoxynucleotide terminators such that the specific nucleotide at the position of the mutation may be easily determined via a colorimetric assay.

Transgenic Animals and Cell Lines

Having identified subjects having a predisposition to Parkinson's disease associated with a specific mutation, the subjects can participate in the screening of putative agents capable of treating Parkinson's disease. This method comprises administering the test agent to the subject, which may be a human, which has a mutation in a gene associated with Parkinson's disease and monitoring the effect of the agent on the subject's condition. If the symptoms of Parkinson's disease improve, the agent can be used as a treatment for the disease.

In addition, it is possible to develop transgenic model systems and/or cell lines containing the mutated nucleic acid(s) for use, for example, as model systems for screening for drugs and evaluating drug efficiency. Additionally, such model systems provide a tool for defining the underlying biochemistry of, for instance, the mutated synuclein gene, thereby providing a rationale for drug design.

One approach to creating transgenic animals is to mutate the animal gene of interest by in vivo mutagenesis, transfer the mutant gene into embryonic stem cells by DNA transfection and inject the embryonic stem cells into blastocysts in order to retrieve offspring which carry the disease-causing mutation (31). Alternatively, the technique of microinjection of the mutated gene, into a one-cell embryo followed by incubation in a foster mother can be used. Alternatively, viral vectors, e.g., Adeno-associated virus, can be used to deliver the mutated gene to a stem cell, or may be used to target specific cells of a fully developed animal (32,33).

Antibodies and Recombinant Expression of Polypeptides

When the mutated gene product is a polypeptide, e.g. the 209 mutation, it can be used to prepare antisera and monoclonal antibodies using, for example, the method of Kohler and Milstein (34). Such monoclonal antibodies could then form the basis of a diagnostic test, or may even be useful in therapies directed toward inhibiting the action of the mutant protein in a patient with Parkinson's disease.

Mutant polypeptides can also be used to immunize an animal for the production of polyclonal antiserum (35). For example, a recombinantly produced fragment of a variant polypeptide can be injected into a mouse along with an adjuvant so as to generate an immune response. Murine immunoglobulins which specifically bind the recombinant fragment can be harvested from the immunized mouse as an antiserum, and may be further purified by affinity chromatography or other means. Additionally, spleen cells are harvested from the mouse and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells, which can then be screened for clones that secrete immunoglobulins which bind the recombinantly produced fragment with an increased affinity. More specifically, immunoglobulins that selectively bind to the variant polypeptides but poorly or not at all to wild-type polypeptides are selected, either by pre-absorption with wild-type proteins or by screening of hybridoma cell lines for specific idiotypes that bind the variant but not wild-type.

These antibodies can be used to screen protein and tissue samples for the presence of mutated proteins. A colored

enzymatic reaction occurs when the specific antibody remains bound to its target protein, in situ, after thorough washing, as directed by established protocols.

Gene expression

The nucleic acid sequences of the present invention will be capable of expressing the desired mutant or normal polypeptides in an appropriate host cell. For expression in host cells, the DNA sequences of the present invention will be operably linked to, i.e., positioned to ensure the functioning of, an expression control sequence. For example, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts. In addition, the DNA sequence of the present invention may also be fused such that the reading frame is conserved to an appropriate signal sequence to facilitate export of the encoded protein across the cell membrane.

Expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. A variety of suitable expression vectors are disclosed in Sambrook et al. (13). Commonly, expression vectors will contain selection markers, e.g., tetracycline resistance or hygromycin resistance, to permit detection and/or selection of those cells transformed with the desired DNA sequences.

E. coli is one prokaryotic host that is particularly useful for cloning and expression of the DNA sequences of the present invention because of the wide variety of available

expression systems. Vectors suitable for use in E. coli are known and are commercially available, i.e. pBR322 (13), pBLUESCRIPT (Stratagene), etc. Also, a variety of different types of expression systems may be used, including plasmids, cosmids, bacteriophage lambda, etc. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. Expression vectors for use in prokaryotic host cells will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any of a variety of well-known promoters may be used, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. A promoter may optionally contain an operator sequence for regulatable gene expression, and will have a ribosome binding site sequence for the initiation of translation.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (36). Vectors for use in eukaryotic cells are known and commercially available, i.e. pcDNA3 (Invitrogen). Eukaryotic cells are actually preferred, and a number of suitable host cell lines capable of secreting intact human proteins have been developed in the art, including CHO cells, COS cells, HeLa cells, myeloma cell lines, Jurkat cells, etc. Promoters for use in eukaryotic vectors may be cell-specific, or capable of being expressed in a wide variety of cells, i.e. viral promoters.

Expression vectors of the present invention (e.g., comprising nucleic acid sequences encoding a mutant or normal polypeptide) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts.

Kits

The method lends itself readily to the formulation of test kits which can be utilized in diagnosis. Such a kit would comprise a carrier compartmentalized to receive in close confinement one or more containers wherein a first container may contain suitably labeled DNA probes. Other containers may contain reagents useful in the localization of the labeled probes, such as enzyme substrates. Still other containers may contain restriction enzymes (such as *Tsp*45I), buffers, etc., together with instructions for use.

DESCRIPTION OF THE INVENTION

Detailed Description of the Preferred Embodiments

The following laboratory procedures were used:

DNA samples were collected upon informed consent. High molecular weight genomic DNA was isolated from whole-blood lysate by methods previously described (38). Genotyping was performed as previously described (39). Pairwise linkage analysis was performed using the MLINK program of the FASTLINK package (40-42). Allele frequencies were used as

reported in the Genomic Data Base (http://gdbwww.gdb.org) and the Cooperative Human Linkage Consortium (CHLC) database (http://www.chlc.org). Multipoint analysis was performed using the LINKMAP program of the FASTLINK package. For the multipoint analysis allele frequencies were set to 1/n where n equals the number of alleles observed. In the two point analysis LOD scores were calculated for both the reported and the 1/n allele frequencies with minimal effect on the maximum LOD score observed. Simulations of multipoint analysis in a subset of the pedigree with different allele frequencies similarly indicated no significant effect on the scores calculated. Maximum LOD scores as shown were observed for the heterozygote and homozygote disease allele penetrance set to 0.99, which is similar to the PD allele penetrance previously reported ranging from 0.88 to 0.94 (3). All unaffected individuals used in the study were of age above the mean for onset of illness. Disease allele frequency was set to 0.0001. Distances and order of genetic markers were set as reported in the CHLC database. Overlapping three point analysis was performed for markers *D4S2361, D4S1647, D4S421* and the PD locus. The 12 allele *D4S2380* locus was not included because of prohibitive time run. Multipoint analysis was performed on an IBM SP2 parallel computer and the SGI Challenge machine.

For mutation analysis genomic DNA was amplified with oligonucleotides (3): 5' GCTAATCAGCAATTTAAGGCTAG 3' (SEQ ID NO 2) and (13): 5' GATATGTTCTTAGATGCTCAG 3' (SEQ ID NO 3) of genbank ID: U46898, under standard PCR conditions. Sequence analysis was performed using the Perkin Elmer dye terminator

cycle sequencing kit on an ABI 373 fluorescent sequencer (ABI, Foster City, CA). Restriction digestion was performed following the PCR with *Tsp*45 I according to manufacturer's protocol (New England Biolabs, Beverly, MA). The digested PCR products were electrophoresed on a 6% Visigel (Stratagene, La Jolla, CA), and visualized by ethidium bromide staining. Pedigree structure in Figure 2 has been slightly modified in order to protect patient confidentiality.

Total RNA was extracted from the lymphoblastoid cell line of an affected individual and first strand synthesis was performed by oligo dT priming (Gibco BRL, Gaithersburg, MD). Primers (1F) 5' ACGACAGTGTGGTGTAAAGG 3' (SEQ ID NO 9) and (13R) 5' AACATCTGTCAGCAGATCTC 3' (SEQ ID NO 10) corresponding to nucleotides 21-40 and 520-501 of genbank L08850 were used to amplify a product of 500 bp containing the mutation at nucleotide 209. PCR products were subjected to restriction digestion by *Tsp*45 I. The mutation at nt 209 creates a novel *Tsp*45 I site (Figure 1), so that the normal allele will be restricted in 4 fragments of 249, 218, 24 and 9 bp, where the mutant allele will have 5 fragments of 249, 185, 33, 24 and 9 bp of size, as shown in Figure 3. Size standards used, were the 100 bp ladder (Gibco BRL, Gaithersburg, MD).

Example 1

In an effort to identify a genetic locus responsible for Parkinson's disease, we performed a genome scan in a large kindred of Italian descent with pathologically confirmed PD (Figure 5). The kindred originated in the town of Contursi

in the Salerno province of Southern Italy (3). Some members emigrated to the United States, Germany and other countries. The extended family pedigree consists of 592 members with 60 individuals affected by PD. The average age of onset for the illness in this pedigree (Figure 5) has been shown to be 46 Å 13 years. One hundred and fourty genetic markers were typed in this pedigree at an average spacing of about 20 cm. Genetic markers at the cytogenetic location *4q21-q23* were the only ones to show linkage to the disease phenotype with a Zmax=6.00 at theta=0.00 for marker *D4S2380I* (see Table 1).

Table 1. Two point LOD scores between chromosome 4q markers and the PD locus

Locus	Two-point LOD scores at recombination fractions of: 0.00	0.01	0.05	0.10	0.20	0.30	0.40	Z_{max}	θ_{max}
D4S2361	-5.60	-0.83	0.30	0.54	0.43	0.21	0.06	0.55	0.12
D4S2380	6.00	5.90	5.30	4.60	3.00	1.50	0.50	6.00	0.00
D4S1647	5.22	5.07	4.47	3.71	2.26	1.05	0.30	5.22	0.00
D4S421	-2.42	0.45	0.77	0.65	0.38	0.22	0.09	0.77	0.05

Recombinations between the disease phenotype and genetic markers were observed in the proximal region for marker *D4S2361* and in the distal region for marker *D4S421*. Genetic markers *D4S2380* and *D4S1647* showed no obligate recombination events in the affected individuals.

Multipoint LOD score analysis between markers *D4S2361*-13cM-*D4S1647*-3cM-*D4S421* and the disease locus places the PD gene between markers *D4S2361* and *D4S421* at a recombination distance of 0.00 cM from marker *D4S1647* with a Zmax=6.04 (Figure 6). This location is favored from the alternative genetic intervals by a difference in the LOD score of greater than three LOD units.

Although expansions of unstable trinucleotide repeats are found in a number of human neurogenerative conditions, there is no evidence for an association of a CAG trinucleotide repeat expansion in families with PD (43). In addition, genetic linkage studies in other families with PD-like illnesses do not support the involvement of several candidate genes (glutathione peroxidase, tyrosine hydroxylase, brain-

derived neurotrophic factor, catalase, amyloid precursor protein, CuZn superoxide dismutase and debrisoquinone 4-hydroxylase) in the etiology of the disorder (44). Genes previously mapped in the general region of linkage include the loci for alcohol dehydrogenase, formaldehyde dehydrogenase, synuclein, UDP-N-acetylglycosamine phosphotransferase and others.

Our localization of a PD susceptibility gene represents the first genetic locus linked to PD. Other distinct clinicopathological entities associated with parksonian features are probably linked to other genetic loci. For example, Wilhelmsen-Lynch disease (disinhibition-dementia-parkinsonian-amyotrophy complex) is linked to the *17q21-q22* chromosomal region (45). If the pathogenesis of diseases affecting the nigrostriatal pathway includes environmental influences, then a range of mutations affecting vulnerable sites in the electron transport chain or enzyme polymorphisms influencing neurotoxin metabolism may vary the penetrance of PD by altering an individual's resistance to exogenous or endogenous agents. However, our finding of a highly penetrant genetic locus linked to PD suggested that abnormalities of a single gene may be sufficient to cause Parkinson's disease.

Example 2

In an effort to identify a specific gene between markers *D4S2361* and *D4S421* that is associated with predisposition to Parkinson's disease, we conducted sequence analysis of candidate genes in this region.

Alpha synuclein, a presynaptic nerve terminal protein, was originally identified as the precursor protein for the NAC peptide, a non beta amyloid component of Alzheimer's disease (AD) amyloid plaques (4). The human alpha synuclein gene was previously mapped in the 4q21-q22 region (5). We refined the mapping, and determined that the alpha synuclein gene is located within the non-excluded region harboring the PD gene in the Italian kindred. Thus alpha synuclein represented an excellent candidate locus for PD.

Sequence analysis of the fourth exon of the alpha synuclein gene revealed a single base pair G209A change from the published sequence of the gene (GenBank ID L08850), which results in an Ala53Thr substitution and the creation of a novel *Tsp*45 I restriction site (Figure 1). Mutation analysis for the G209A change in the Italian kindred shows complete segregation with the PD phenotype with exception of individual 30 (Figure 2), who is affected but not carrying this mutation. This individual apparently inherited a different PD mutation from his father, as we have shown that he shares a genetic haplotype with his unaffected maternal uncle, individual 3, for genetic markers in the PD linkage region.

The frequency of this variation was studied in two general population samples, one consisting of 120 chromosomes of the parents of the CEPH reference families, and the other consisting of 194 chromosomes of unrelated individuals from the blood bank in Salerno, Italy, a city near the town from which the family originated. Of these 314 general population chromosomes none was found to carry the G209A mutation.

Fifty two patients of Italian descent with sporadic PD were also screened for the mutation (Figure 2), along with 5 probands from previously unpublished Greek families with PD. The Ala53Thr change was found to be present in three of the Greek kindreds and it segregated with the PD phenotype. In those three Greek kindreds it is worth noting that the age of onset for the disease is relatively early, ranging from the mid 30's to the mid 50's. Extended haplotype analysis of the Greek kindreds and the Italian PD family suggests that the mutations arose independently on different ancestral chromosomes. The finding of the Ala53Thr substitution in four independent PD families and its absence from 314 control chromosomes provides the strongest genetic evidence that this mutation in the human alpha synuclein gene is causative for the PD phenotype in these families.

We have also demonstrated by RT PCR that the mutant allele is transcribed in the lymphoblast cell line of an affected individual from the Italian kindred (Figure 3) (7). Thus, it is reasonable to assume that the mutant protein is indeed expressed.

Example 3.

Since homologous genes that are related to the alpha synuclein protein have been identified in other species, it seemed reasonable to assume that homologues of alpha synuclein would exist in humans as well. In fact, human beta synuclein has previously been described (46), and is approximately 60% similar to alpha synuclein at the protein level.

We set out to identify other related homologues by searching various databases for homologous genes and proteins. Protein sequence databases searched included the NR (non-redundant) and "month" databases of Genbank and Swiss Prot. Nucleotide databases included NR, month, dbstf, GSS (Genome Sequence Service) and EPD (eurkaryotic Promoter Database). Several human clones were identified and characterized as alpha, beta and gamma clones as shown in Figure 7. Potential gamma clones were identified on the basis of homology to known rat and mouse sequences. Although gamma synuclein has been identified in species other than human, this is the first identification of the corresponding gamma synuclein from humans.

Using two primers sets designed from known database sequences (5'ATGTCTTCAAGAAGGGCTTC3'; 5'CCTTGGTCTTCTCAGCTGCT3' and 5'AGCGTGGATGACCTGAAGAG3'; 5'AGCACAGGTGGACAGGCCAAG3'), we have isolated two BAC clones, 139A20 and 174P13, from a Genome System commercial Bacterial Artificial Chromosome library (St. Louis, MO) which contain the human beta and gamma synuclein genes, respectively. The beta gene contained one clone 139A20 has been sequenced as shown in Figure 8 (SEQ ID NO 11), which contains all coding exon sequences and some additional non-coding intronic sequence. The gamma clone 174P13 has been sequenced and is available in GenBank: accession number AF044311. Sequence from the 5' end is given in Figure 9 (SEQ ID NO 12), and sequence from the 3' end is given in Figure 10 (SEQ ID NO 13). The human alpha synuclein gene has also been sequenced as shown in Figure 11, which provides the sequence of each separate exon region with

some additional flanking intronic sequence for each exon. (SEQ ID NOs 14-19)

The three human homologues are highly conserved at the protein level. The alpha and beta human homologues have about 60.4% similarity. And the gamma homologue is about 38.3% and 32.8% similar to the alpha and beta homologues, respectively, based on the portion of the coding sequence that we have obtained thus far. Thus, it is reasonable to presume that mutations in either the beta or gamma synuclein gene may also result in Parkinson's disease.

References

Each of the following citations is herein incorporated by reference:

U.S. PATENTS

5,494,794	Wallace	February 27, 1996
5,047,519	Hobbs, Jr. et al.	September 10, 1991

OTHER REFERENCES

1. J. Parkinson, *An Essay on the Shaking Palsy* (Whitingham and Rowland, London, 1817); W.R. Gowers, *A Manual of Diseases of the Nervous System*, 2nd ed. (Blakinston, Philadelphia, 1893), pp.6366-6657.

2. A.M. Lazarrini et al., *Neurology* **44**, 499 (1994).

3. L.I. Golbe et al., *Ann. Neurology* **27**, 276 (1990); M.H Polymeropoulos et al., *Science* **274**,1197 (1996).

4. K.Uèda et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11282 (1993).

5. X. Chen et al., *Genomics* **26**, 425 (1995);Y. Shitasaki et al., *Cytogenet. Cell. Genet.* **71**, 54 (1995).

6. A. Iwai et al., *Biochemistry* **15**, 10139 (1995); P.H. Weinreb et al., *Biochemistry* **29**, 13709 (1996); P.H. Jensen et al., *Biochem J* **15**, 91(1995); P.H. Jensen et al., *Biochem. J.* **323,** 539 (1997).

7. L. Maroteaux and R.H. Scheller, *Brain Res. Mol. Brain. Res.* **11**, 335 (1991).

8. R.L. Doty et al., *Ann. Neurol.* **32**, 97 (1992); R.K. Pearce, C.H. Hawkes, S.E. Daniel *Mov. Disord.* **10**, 283 (1995).

9. J.M. George et al., *Neuron* **15**, 361 (1995).

10. L. Maroteaux, J.T. Campanelli, R.H. Scheller *J. Neurosci.* **8**, 2804 (1988).

11. Alzheimer's Disease Collaborative Group *Nature Genet.* **11**, 219 (1995); J. Perez-Tur et al., *NeuroReport* **7**, 297 (1995); R. Sherrington et al., *Nature* **375**, 754 (1995); S. Sorbi et al., *Lancet* **346**, 439 (1995); W. Wasco et al., *Nature Med* **1**, 848 (1995); E. Levy-Lahad et al., *Science* **269**, 970 (1995); E. I. Rogaev et al., *Nature* **376**, 775 (1995).

12. Hsiao K et al., *Nature* **338**, 342 (1989).

13. Maniatis et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

14. Berger and Kimmel (1987) "Guide to Molecular Cloning Techniques,"Methods in Enzymology, Volume 152, Academic Press, Inc., San Diego, Calif.

15. Gibbs et al. (1989) Nucleic Acids Res. 17:2437.

16. Kwok et al. (1990) Nucleic Acids Res. 18:999.

17. Miyada et al. (1987) Methods Enzymol. 154:94.

18. Dihella et al. (1988) Lancet 1:497.

19. Promega Protocols and Applications Guide (1991) Promega Corporation, Madison, Wis.

20. Orita et al. (1989) Genomics 5:874-879.

21. Orita et al. (1990) Genomics 6:271-276.

22. Newton et al. (1989) Nucleic Acids Res. 17:2503.

23. Nichols et al. (1989) Genomics 5:535.

24. Okayama et al. (1989) J. Lab. Clin. Med. 114:105.

25. Sarkar et al. (1990) Anal. Blochem. 186:64.

26. Sommer et al. (1989) Mayo Clin. Proc. 64:1361.

27. Wu (1989) Proc. Natl. Acad. Sci. (U.S.A.) 86:2757

28. Dutton et al. (1991) Biotechniques 11:700.

29. Baany et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88:189.

30. R. A. Weiss (1991) Science 254:1992.

31. Frohman and Martin, Cell (1989) 56:145.

32. Mendelson et al. Virology 166:154-165.

33. Wondisford et al. (1988) Molec. Endocrinol. 2:32-39 (1988).

34. Kohler and Milstein, (1975) Nature 256:495-497.

35. Antibodies: A Laboratory Manual, Harlow and Lane (1988), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

36. Winacker (1987) "From Genes to Clones," VCH Publishers, New York, N.Y.).

37. Quenn et al. (1986) Immunol. Rev. 89:49-68.

38. Bell et al. (1981) Proc. Natl. Acad. Sci. USA 78: 5759.

39. Gyapay et al. (1994) Nature Genet. 7: 262.

40. Lathrop et al. (1984) Proc. Natl. Acad. Sci USA 81: 3442.

41. Cottingham et al. (1984) Am. J. Hum. Genet. 54: 252.

42. Gupta et al. (1995) Comp. Biomed. Res. 28: 116.

43. Carrero-Valenzuela et al. (1995) Neurology 45: 1760; M.H. Polymeropoulus, data not shown.

44. Gasser et al. (1994) Ann. Neurol. 36: 387.

45. Lynch et al. (1994) Neurology 44: 1878.

46. Jakes et al. (1994) Febs Letts. 345: 27-32.

47. Polymeropoulos et al. (1997) Science 276:2045-2047, which is relied upon and hereby expressly incorporated by reference herein.

48. Lavedan et al. (1998) in press, which is relied upon and hereby expressly incorporated by reference herein.

49. This application is based on provisional application number 60/505,684 filed June 25, 1997 which is relied upon and hereby expressly incorporated by reference herein.